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Per the Official Action of 11/28/07, attached is a copy of the translation of Fu, et al. (1994) Zhonghua Wai Ke Za Zhi, 32(10): 615-18.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Robert M. Kelly, Art Unit 1633, whose telephone number is (571) 272-0729. The examiner can normally be reached on M-F, 9:00am-5:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Joseph Woitach can be reached on (571) 272-0739. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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RELATIONSHIP BETWEEN GUT ORIGIN BACTERIA AND WOUND INFECTION AFTER
THERMAL INJURY

[Yan zhong shao shang hou chang dao xi jun yu chuang mian gan ran di guan xi]

Fu Wei-ling et al.

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RELATIONSHIP BETWEEN GUT ORIGIN BACTERIA AND WOUND INFECTION AFTER THERMAL INJURY

[Yan zhong shao shang hou chang dao xi jun yu chuang mian gan ran di guan xi]

Fu Wei-ling et al.

Institute of Burns Research, Southwestern Hospital, Third Military Medical College, Chongqing

Zhong Hua Waike Zazhi (Journal of Chinese Association of Surgeons)

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ABSTRACT

Relationship between gut origin bacteria and wound infection after thermal injury (Fu Wei-ling, Xiao Guang-xia, Yu Pei-wu, et al. (Institute of Burns Research, Southwestern Hospital, Third Military Medical College, Chongqing 630038.

The pUC19 plasmid vector trace with restriction map analysis and fluorescence labelling bacteria method were applied to study the relationship between the gut origin bacteria and wound infection. According to the characteristic of pUC19 plasmid, a special animal model was designed. 110 Wistar rats received 30% TBSA full thickness burns. On hours 6, 12, 24, 48 and day 12 postburn, injured animal were killed. Subeschar tissue homogenates were examined under fluorescence microscope, and bacterial culture, isolation of plasmids and restriction map analysis were also carried out. The results show that during early stage of burns, 32.5% of fluorescence labelling bacteria and 10.81% of pUC19 plasmid vectors could be detected from the subeschar specimens. 12 day postburn, the detectable rage of pUC19 plasmid vector increased to 62.5%. Beside the factor of early colonization, the contaminative route from gut perineum and then wounds should be considered.)

Keywords:

Burn wound

Infection

Wound infection

Burn wound infection plays a very important role in burn infection [1]. Enterogenous infection is receiving much more attention accompanying the research progress regarding the translocation of fecal organisms [2]. Based on the fact that the burn wound infectious bacteria and the fecal organisms are often consistent, scientists have proposed a very interesting subject with respect to the relationship between translocation infection by fecal organisms and burn wound infection. Said subject still remains at the stage of perceptual knowledge due to a lack of ideal animal models and of convincing experimental techniques. Accordingly, we designed an animal model to observe the dynamics of fecal organisms and burn wound organisms in attempts to investigate the relationship between translocation infection by fecal organisms and burn wound infection more precisely.

Materials and methodology

1. Labeling substance: (1) pUC19 plasmid vector (Promega Company) was amplified in LB medium containing 100 µg/mL ampicillin. (2) Fluorescence-labeled bacteria: 1/10000 acridine orange was added to *E. coli* solution in CMCC 44102 solution, which was incubated by shaking overnight at 37°C.
2. Restriction DNA endonuclease: Hind III, ECORI, products of HUA MEI Company.
3. Extraction of plasmid was in accordance with Kado's method.
4. Experimental animals: 110 Wistar rats, body weight 225.87 ± 32.64 g. The experiment was divided into two parts for fluorescence-labeled bacteria observation and pUC19 trace observation, respectively. Four time points, namely 6, 12, 24 and 48 h, were established for the part of fluorescence-labeled bacteria observation. Each time point group consisted of 10 animals and the control group

consisted of 10 animals, a total of 50 animals. The same grouping as in the aforementioned fluorescence labeling group was performed for the pUC19 plasmid trace group with an additional observation group on day 12 postburn (10 rats), a total of 60 animals.

5. Experimental procedure: (1) fluorescence-labeled group: 2 mL acridine orange fluorescence-labeled bacterial solution (bacterial count 10^9 /mL) were injected in Wistar rats through a gastric catheter. After 8 h, the rats were anesthetized with 1% sodium barbital by intraperitoneal injection, followed by causing 30% TBSA third degree burns, and 3 mL/100 g body weight of sterilized saline solution was injected intraperitoneally immediately afterward. The rats were sacrificed at 6, 12, 24 and 48 hours postburn. Intestinal membranous lymph nodes, livers and subeschar tissue were excised under sterilize conditions, and the organs were homogenized and divided into 2 parts, respectively. One part was utilized for quantitative culture of bacteria and the other part was utilized for determining the total bacterial count of 10 observation fields under a fluorescence microscope (Olympus VANOX) at 10 x 100 magnification, and the average count of each field was determined. (2) pUC19 plasmid trace group: The drinking water for the animals was prepared as an aqueous solution containing 300 µg/mL ampicillin, which was supplied for 3 days consecutively to "cleanse" the intestinal tract. Bacteria (bacterial count 10^8 /mL, 2 mL/rat) carrying pUC19 plasmid vector were inoculated in the intestinal tract of the animals through a catheter. The animals were given a liquid containing 100 µg/mL ampicillin for 3 days. After the pUC19 plasmid vector was confirmed to be incorporated in the intestinal tract by culturing the feces in LB culture medium containing 100 µg/mL ampicillin, 30% TBSA third degree burns were caused on the backs of the animals. The animals were sacrificed at hours 6, 12, 24, 48 and day 12 (same method as before). The homogenized intestinal membranous lymph nodes, liver and subeschar tissues were cultured at 37°C for 18 h by shaking in LB culture medium containing ampicillin. The plasmid was extracted in accordance with Kado's method from those showing positive culture. The

extracts were digested with restriction endonucleases ECOR I and Hind III for 1 h in low-salt and high-salt buffers respectively. Electrophoresis with 1% agar was conducted, followed by ethidium bromide-nucleic acid staining, and the result was observed under long-wavelength UV light.

Results

1. Fluorescence bacterial labeling assay: The labeled bacteria in the intestinal tract passed the damaged intestinal mucous membrane after a severe burn and penetrated into intestinal membranous lymph nodes and broke the clearance function of the liver to reach the subeschar tissue. Live bacteria exhibit green fluorescent light while dead bacteria exhibit orange-red fluorescent light in the image of a fluorescent microscope (Figures 1-3).

The detection rates of the labeled bacteria under microscope were in the order of intestinal membrane lymph nodes, liver and subeschar tissue (Table 1). Table 2 shows the detection rates and bacterial numbers of the labeled bacteria under microscope at various time points.

Table 1: Detection rates of fluorescence-labeled bacteria in homogenates

| | | ② | ③ | ④ |
|---|--------|-----|-----------|-----------------|
| ① | 脏 器 | 标本数 | 阳性率 (%) | 荧光菌数/视野 平均值* |
| ⑤ | 肠系膜淋巴结 | 40 | 38 (95.0) | 3.8 |
| | 肝脏组织 | 40 | 23 (57.5) | 2.1 |
| | 皮下组织 | 40 | 13 (32.5) | 1.1 |

*Mean value of 10 fields under an oil immersion lens

- Key:
- 1

Organ
- 2

Number of specimens
- 3

Positive rate
- 4

Fluorescent bacteria number/field

Mean value

5 Intestinal membranous lymph node

Liver

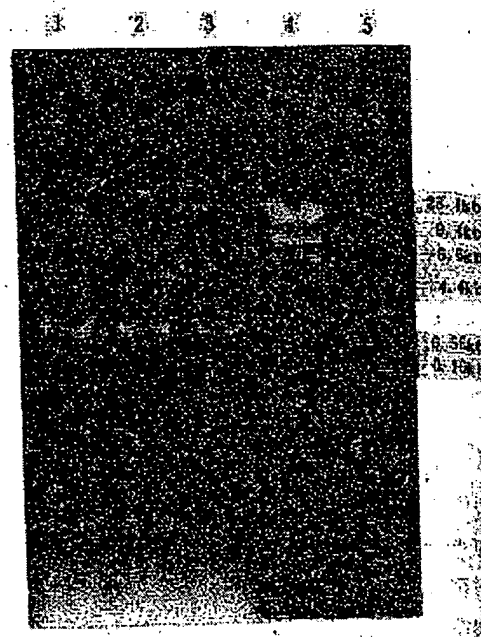
Subeschar tissue

Table 2: Results of microscope examination of fluorescence-labeled bacteria
at various time points postburn

| ① 组别 | ② 肠系膜淋巴结 | | ③ 肝 | | ④ 皮下 | |
|--------|----------|-------------------|----------|-------------------|----------|-------------------|
| | ⑤ 检出率(%) | ⑥ 菌量(cfu/g) | ⑤ 检出率(%) | ⑥ 菌量(cfu/g) | ⑤ 检出率(%) | ⑥ 菌量(cfu/g) |
| ⑦ 烧伤给菌 | | | | | | |
| 6小时 | 100 | 3.0×10^4 | 70 | 2.1×10^4 | 40 | 1.4×10^4 |
| 12小时 | 100 | 5.5×10^4 | 70 | 3.1×10^4 | 40 | 2.5×10^4 |
| 24小时 | 90 | 7.4×10^4 | 50 | 2.6×10^4 | 30 | 9.0×10^3 |
| 48小时 | 70 | 8.2×10^3 | 40 | 1.5×10^3 | 20 | 5.0×10^2 |
| ⑨ 正常给菌 | 40 | 8.0×10^3 | 0 | 0 | 0 | 0 |

- Key:
- 1 Group
 - 2 Intestinal membranous lymph nodes
 - 3 Liver
 - 4 Subeschar
 - 5 Detection rate
 - 6 Bacterial count
 - 7 Burned bacteria-fed
 - 8 Hour
 - 9 Normal, bacteria-fed

2. Using pUC19 plasmid trace: Ampicillin-resistant bacteria could be detected from the intestinal membranous lymph nodes, liver and subeschar tissue postburn. Extraction of plasmid DNA and analysis by restriction map showed that the ampicillin-resistant bacteria isolated from the intestinal tract and under-scar tissue had the same plasmid DNA restriction map (Figure 4). Table 3 shows the positive incubation rates of the pUC19 plasmid vector and numbers at various time points postburn. Table 4 shows the detection rates of the ampicillin-resistant bacteria in the early and later postburn stages.



1. Ampicillin-resistant cell isolated from subeschar tissue; 2. Ampicillin-resistant cell isolated from intestinal tract; 3. Standard pUC19 plasmid bacteria; 4. Hind III spliced DNA; 5. Control

Figure 4: Bacterial plasmid restriction map

Table 3: pUC19 plasmid vector bacterial counts and positive rates

at various time points after severe burn

| ① 组别 | ② 肠系膜淋巴结 | | ③ 肝脏组织 | | ④ 皮下组织 | |
|--------|-------------------|----------|-------------------|----------|-------------------|----------|
| | ⑤ 菌量(cfu/g) | ⑥ 阳性率(%) | ⑤ 菌量(cfu/g) | ⑥ 阳性率(%) | ⑤ 菌量(cfu/g) | ⑥ 阳性率(%) |
| ⑦ 烧伤组 | | | | | | |
| 6小时 | 5.0×10^4 | 89 | 1.3×10^3 | 60 | 7.1×10^5 | 11 |
| 12小时 | 4.0×10^4 | 80 | 1.4×10^3 | 60 | 1.5×10^4 | 10 |
| 24小时 | 3.1×10^4 | 80 | 1.6×10^3 | 50 | 4.0×10^2 | 10 |
| 48小时 | 4.0×10^3 | 62.5 | 7.5×10^2 | 37.5 | 3.1×10^2 | 12.5 |
| 12天 | 5.1×10^3 | 25 | 0 | 0 | 0 | 0 |
| ⑩ 正常对照 | 1.0×10^3 | 20 | 0 | 0 | 0 | 0 |

- Key
- 1 Group
 - 2 Intestinal membranous lymph nodes
 - 3 Liver tissue
 - 4 Subeschar tissue
 - 5 Bacterial count
 - 6 Positive rate
 - 7 Burn group
 - 8 Hour __
 - 9 Day __
 - 10 Normal control

Table 4: Comparison of detection of pUC19 plasmid in various organs
at various time points in early and later stages of burn

| ① 脏 器 | ② 烧 伤 早 期 | | | ③ 烧 伤 迟 期 | | |
|----------|-----------|-------|----------|-----------|-------|----------|
| | ④ 标本数 | ⑤ 阳性数 | ⑥ 阳性率(%) | ④ 标本数 | ⑤ 阳性数 | ⑥ 阳性率(%) |
| ⑦ 肠系膜淋巴结 | 37 | 39 | 76.4 | 3 | 1 | 12.5 |
| 肝脏组织 | 37 | 19 | 51.4 | 3 | 0 | 0 |
| 皮下组织 | 37 | 4 | 10.8 | 3 | 5 | 62.5 |

Early stage means within 48 h of burn; later stage means 288 h after burn (12 d)

- Key
- 1 Organ
 - 2 Early stage of burn
 - 3 Later stage of burn
 - 4 Number of specimens
 - 5 Positive number
 - 6 Positive rate
 - 7 Intestinal membranous lymph nodes
 - Liver tissue
 - Subeschar tissue

Discussion

Research reports on enterogenous infection have increased gradually in recent years. Many scientists in the academy have validated [4, 5] that enterogenous infection is an important pathway for burn wound infection. The issue that follows is how to elucidate the relationship between enterogenous infection and burn wound infection. Can intestinal bacteria reach the wound in the early stage of burn? Can they reach to the subeschar tissue and become latent infectious factors for the burn wound? The questions also include whether the burn wound in the later stage is still related to enterogenous infection.

There are still no convincing reports on these questions due to the limitations in the experimental conditions, animal model as well as some traditional conceptual limitations. Fluorescence labeling and isotope labeling methods have generally been employed so far for tracking enterogenous infectious bacteria, but some problems remain with respect to non-specificity. Accordingly, searching for a better labeling system and establishing an animal model that conforms to the system are vitally important to investigating the relationship between enterogenous infection and burn wound infection. The pUC19 plasmid was established in the 1980s and has been widely employed in identifying molecular clones and recombinant genes [6]. Said plasmid has the characteristics of (1) carrying an ampicillin-resistant gene, thus the plasmid is capable of expressing anti-ampicillin activity; (2) having restriction sites of restriction endonuclease for polyclones; (3) lacking *nic/bom* site. Therefore, gene transfer can be performed through the binding action of bacteria. Said characteristics make pUC19 plasmid an excellent labeling substance for investigating the translocation of intestinal tract bacteria. An animal model can be established for residing trace bacteria in the intestinal tract and screening the translocated bacteria in target organs by using the non-transfer property of pUC19 plasmid among bacteria and ampicillin antibodies. The homogenous nature of the translocated bacterial strains can be verified by the analytical method with restriction fingerprint mapping by using its characteristic of polyclone restriction site. The animal model designed in the present trace experiment basically solves the problems of non-specificity with other labeling methods. The method also provides better reproducibility and accuracy.

Comparison of the two methods utilized in the present experiment showed that the detection rate (32.5%) of the subeschar fluorescence-labeled bacteria in burned animals was significantly higher than that (10.81%) by pUC19 plasmid trace method. Based on our experience in the studies, certain false positive reactions can occur from mutual staining between fluorescence-labeled bacteria and non-fluorescence-labeled bacteria and from non-specific fluorescence in tissues. Individual differences

among the subjects observed is one of the factors affecting the results. Also, fluorescence diminishes rapidly due to growth, metabolism and death of bacteria, which is disadvantageous for long-term observations. Accordingly, the method with fluorescence-labeled bacteria can be utilized for qualitative investigations of bacteria translocating from the intestinal tract to subeschar tissue in the early burn stage, but using it for quantitative observation or long-term investigation is still unfeasible.

About 10.8% of the bacteria in the intestinal tract broke through the barrier of the liver phagocytes and were dispersed to subeschar tissue in the early stage of severe burn when the animal model with pUC19 plasmid vector was utilized. Bacteria translocate easily to locations with localized burn damage. The bacterial count in the liver decreases gradually due to the clearance by the organic immune system as the barrier function of the intestinal tract gradually recovers, but the bacteria in the subeschar tissue show no significant change. The detection rate of the labeled bacteria increased again in the wound in the later stage of the burn (scar dissolving stage). The possibility of the pUC19 plasmid vector bacteria in the wound coming from fecal waste should also be taken into consideration at this point.

In summary, the endogenous intestinal bacteria can penetrate through the damaged intestinal membranous barrier and disperse in the internal organs in the early stage of a severe burn, and the possibility has been validated by many studies. The present investigation using the pUC19 plasmid vector validated that translocation of intestinal bacteria could disperse to the subeschar tissue in the early stage of a burn. Reports showed that the origin of at least 30% of sepsis in early burn stages could not be identified. For these cases, the possibility of enterogenous infection should be taken into consideration.

(Figures 1-3 of this article are shown on page 107)

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